SHORT-TERM EARLY LIFE STAGE GROWTH TEST USING SACFRY AND EARLY SWIM-UP STAGES OF RAINBOW TROUT *(ONCORHYNCHUS MYKISS)*

METHOD DEVELOPMENT AND DATA INTERPRETATION ILLUSTRATED BY EXPOSURE TO COPPER, SODIUM DODECYL SULPHATE, 2,4,5-TRICHLOROPHENOL AND 3,4-DICHLOROANILINE

SECOND EDITION

JULY 1998

Ontario

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ABSTRACT

The late sacfry to early swim-up fry life stage of rainbow trout is the shortest most sensitive period for uptake of toxicants and expression of effects. The Ministry of Environment has developed a simple, cost effective, 12-day growth test using this life stage to provide data on individual chemicals required for the development of Federal and Provincial Water Quality Guidelines to protect salmonids from chronic levels of toxicity, and to monitor possible chronic toxicity effects in non-acutely toxic effluents and receiving waters. Tests during this developmental stage of the protocol using copper, sodium dodecyl sulphate, 2,4,5-trichlorophenol, and 3,4- dichloroanaline have shown the protocol to be as sensitive as longer early life stage (ELS) protocols for estimating chronic effects of metals, surfactants, and organic chemicals in coldwater fish. For some organic chemicals with low absorption rates, and those with narcotic properties, eg. 3,4-dichloroaniline, the exposure period may need to be extended up to 21 days. Any extension period required is determined by simple observations on the degree of yolksac absorption and exogenous feeding compared to the controls during the swim-up fry stage of the test.

RÉSUMÉ

Le stade de l'alevin vésiculé avancé au surnageant (à l'âge de six jours) constitue la période du cycle biologique de la truite arc-en-ciel la plus sensible tant sur le plan de l'absorption des substances toxiques que sur le plan de l'expression des effets. J'ai mis au point un test d'inhibition de la croissance de 12 jours, qui est très simple et peu coûteux et qui fait appel à ce stade du cycle biologique. Le test permet de recueillir les données nécessaires à l'établissement de normes provinciales et fédérales pour différentes substances chimiques en vue de protéger les salmonidés contre une exposition à des niveaux de toxicité chroniques. Il permet également de surveiller les effets des niveaux de toxicité chroniques dans les effluents et les eaux réceptrices à toxicité non aiguë. Les essais effectués lors de la mise au point de la méthode utilisant le cuivre, le dodécyl sulfate de sodium, le 2,4,5 trichlorophénol et la 3,4 dichloroaniline ont montré que la méthode était aussi sensible que d'autres de plus longue haleine faisant appel aux premiers stades larvaires pour l'évaluation des effets chroniques des métaux, des agents tensioactifs et des substances chimiques organiques chez les poissons d'eaux froides. Il s'avère parfois nécessaire de prolonger la période d'exposition à 21 jours dans le cas des substances chimiques organiques à faible vitesse d'absorption, et dans le cas des substances ayant des propriétés narcotiques, par exemple la 3,4 dichloroaniline. L'évaluation de la période d'exposition supplémentaire se fait par la simple observation, durant le stade de l'alevin surnageant, du degré d'absorption de la vésicule vitelline et d'alimentation exogène comparativement à celles des témoins .

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LIST OF ABBREVIATIONS

°C..... degree(s) Celsius
$CaCO_3$..... calcium carbonate
Cu..... copper
cm..... centimetre(s)
c.v..... coefficient of variation
d..... day(s)
DCA..... dichloroaniline
$EC_{50,(20,5)}$..... effective concentration (estimated) for a 50% (20% or 5%) specified effect compared to the control
ELS..... early life stage
fct..... footcandle, a unit of illumination based on units per square foot
g..... gram(s)
h..... hour(s)
HCl..... hydrochloric acid
IC_x..... inhibiting concentrations causing the % differences in wet weight gain of the LOEC and NOEC from the control, in a specified test
$IC_{20,(25)}$..... inhibiting concentrations (estimated) for a 20% (25%) specified effect compared to the control, in a specified test
L..... litre(s)
LC_{50}..... median lethal concentration
LOAEL..... lowest-observed-adverse-effect concentration
LOEC..... lowest-observed-effect concentration
log K_{ow}..... logarithm of the octanol-water partitian coefficient
lx..... lux, a unit of illumination based on units per square metre.
One lux = 0.0929 foot candles, (fct), one foot candle = 10.76 lux.
M..... mole (1 mole of a compound equals its molecular weight in grams)
mg..... milligram(s)
mL..... millilitre(s)
mm..... millimetre(s)
MOEE..... Ministry of Environment and Energy
MSD..... minimum significant difference
n..... number of observations

N..... normal (solution) - an aqueous solution containing 1 equivalent weight in grams of an element or compound, in 1 litre of solution

NaOH..... sodium hydroxide

NH_3..... ammonia

NOEC..... no-observed-effect concentration

p..... probability

pH..... the negative logarithm of the activity of the hydrogen ion concentration ($-\log [H^+]$) of a solution, indicating the degree of acidity or alkalinity in a scale from 1-14. pH 1 = very strongly acidic, pH 7 = neutral, pH 14 = very strongly alkaline

ppm..... parts per million

ppt..... parts per trillion

s.d. ..... standard deviation

SDS..... sodium dodecyl sulphate

s.e. ..... standard error

TCP..... trichlorophenol

U.S.E.P.A. .. United States Environmental Protection Agency

μg..... microgram(s) (1μ = 1/1,000 of a mg)

μg/L..... micrograms per litre

μM..... micromole(s)

LIST OF SYMBOLS

%.....percent

".....inches

≈.....approximately

x.....mean

±.....plus or minus

<.....less than

>.....greater than

=.....equal to

≤.....less than or equal to

≥.....greater than or equal to

INTRODUCTION

A sublethal early life stage test applicable to cold water fish is needed to provide data for the development of guidelines for the protection of the aquatic environment against chronic effects of toxicants. As such, the test must be cost effective yet sensitive to a wide range of chemicals, and interpretation of the biological effects of the test endpoint must be clear. Since it is never possible to accurately extrapolate lab results directly to the field to predict chronic effects, there will always be some degree of uncertainty in estimating the concentrations of toxicants that will cause chronic toxic effects in receiving waters. Therefore, for practical purposes, it is necessary to develop the most cost effective test that has the least degree of uncertainty.

According to the majority of literature reports, the most sensitive stages in ELS tests for absorption of toxicants are the few hours from fertilisation to water hardening and the sacfry stage, whilst the stages that show the greatest effects of the toxicants are hatching, and from swim-up through onset of feeding and early fry development. However, the period between water hardening and hatching is the longest, relatively insensitive ELS stage to toxicants, therefore this stage could be omitted with the least reduction in sensitivity in developing a sublethal short-term ELS toxicity test. Therefore, the test should be based on the late sacfry to early fry life-stages. Evidence to support this has been shown in several studies on rainbow trout and other salmonids.

Sublethal (96h and 10d) and lethal (96h and 7d) exposures have shown that the salmonid late sacfry to swim-up period includes stages of optimal absorption into the larva of neutral and polar organic chemicals and metallic and non-metallic inorganic chemicals, and the stage at which the effects of toxicants become most evident. Daye and Garside (1977) found Atlantic salmon (*Salmo salar*) early cleavage embryos to be less sensitive than sacfry in 7 day

exposures to pH values from 3.0 to 4.0. Rice and Bailey (1980) exposed eyed egg to early fry stages of pink salmon (*Oncorhynchus gorbuscha*) to ammonia in acute lethality tests. Sacfry just prior to emergence were slightly more sensitive than fry at 83 μg/L NH_3 whilst eyed eggs hatched normally in concentrations >1.5 mg/L NH_3. A similar test by Rice et al (1975) showed eggs to be the most resistant and swim-up fry the most sensitive to Prudhoe Bay crude oil (96h LC50 of swim-up fry = 12 ppm). They also reported that late sacfry showed the greatest growth reduction after exposure of early, mid, and late sacfry to 0.73 ppm crude oil for 10d followed by transfer to control water until swim-up. In experiments with rainbow trout, Helder (1981) exposed freshly fertilised eggs and newly hatched sacfry for 96h to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), then transferred them to control water for 5 to 6 months. At 1.0 ppt TCDD (the lowest concentration both groups were exposed to), approximately 2.5% mortality occurred in both groups during the sacfry stage. Of the remaining fry, teratogenic defects occurred in 20% of those exposed as eggs and 5% of those exposed as sacfry. However, growth retardation noted throughout the development of both groups was reduced by ~40% in the sacfry ($p < 0.001$) but only ~5% in the egg exposed group ($p < 0.025$). Van Leeuwen et al. (1985), exposed six early life stages of rainbow trout, including fertilized eggs before (0h) and after (24h) water hardening, early eyed eggs (14d), late eyed eggs (28d), sacfry (42d), and early fry (77d) in 96h LC50 tests using cadmium, manganese ethylenebisdithiocarbamate (maneb), pentachlorophenol, 1,2,4,5-tetrachlorobenzene, parathion, and dieldrin. They reported the early fry stage to be the most critical in all tests. Sacfry were almost as sensitive as early fry to cadmium, maneb and pentachlorophenol. Freshly fertilized eggs were relatively insensitive to all the chemicals tested. In a static bioaccumulation test with dieldrin, the bioconcentration factor increased during embryonic development reaching a maximum during the sacfry stage, then decreased sharply after swim-up as the clearance rate increased. Most dieldrin was accumulated in the yolk, larval concentration remaining low until the yolk was absorbed.

These reports substantiate the choice of the late sacfry to early swim-up fry stage of salmonid development as the most sensitive to use for optimal toxicant uptake and expression of toxicant effects, in a short-term sublethal early life stage test.

Decisions on the length of each life stage to include in the test should take into consideration the age at which test initiation procedures can be successfully carried out, as well as the minimum time necessary for toxicant absorption as well as expression of effects, combined with the most sensitive and easily interpreted endpoint. In a review of 25 years of chronic life cycle and early life stage tests, Woltering (1984) found that fry growth and survival were the most sensitive combined endpoints in 173 tests using a variety of chemical classes and effluents. Survival alone was reported to be more sensitive than growth, but growth was assessed by measuring length and/or weight, even though, in salmonid fry, length is less sensitive than weight as it does not take into account the considerable differences in the depth and breadth of the body. No indication of the method used was included in Woltering's summary tables of the most sensitive responses. Furthermore, Woltering noted that within and between laboratory variability in the growth response considerably reduced its sensitivity. The variability was thought to be partly caused by differences in feeding and in space availability. Growth tests were also longer than tests for survival. Ninety day tests were commonly used for salmonid early life stage growth tests, which usually started during embryogenesis. As length of exposure is the most important criterion affecting cost, Woltering's conclusions that survival was a more useful endpoint than growth was justified at that time.

More recently, Hodson et al. (1991), showed growth to be the most sensitive endpoint in three month, early life stage tests using salmonids, even though its variability increased with the length of exposure during the tests. The growth variability in ELS test protocols developed

until now has been a deterrent in using this endpoint in short-term, salmonid early life stage tests. However, a standardised short-term test with maximised sensitivity of the growth response would reduce the cost and variability found in traditional growth tests. The test that the Ministry of Environment and Energy has developed is a short-term ELS growth test, using the shortest combination of most sensitive life stages in rainbow trout, at sublethal concentration levels ranging from control to incipient mortality. Avoiding short-term mortality allows growth to be the single endpoint, simplifies statistical analysis, increases the test sensitivity, and reduces the uncertainty in estimating chronic levels of toxicity. Rainbow trout eggs for pre-test culturing in the laboratory at the pre-hatch stage are available from certified commercial fish hatcheries for most of the year.

At the sacfry to swim-up fry stage of salmonids individual gain in wet weight must be used as the growth parameter. Even when using same-aged, sibling sacfry, the sacfry weights are still highly variable, (see Figure 1a), hence, to maximise the test sensitivity, it is essential to restrict the range of initial sacfry weights used in the test to ± 10% of the mean, eliminating the outliers among the weighed sacfry, and to use individual wet weight gain during this short-term test as a measure of growth, rather than the final weight alone. Dry weight should not be used, (see below).

It is also essential to include a period of exogenous feeding in the test. During the sacfry stage the dry weight of the total sacfry decreases slightly from hatch to swim-up as the yolksac is absorbed into the larva because not all of the energy from the metabolised yolk[1] can be used for growth and development, some has to be used for respiration and activity, (see Figure 1b). They have no external source of carbon until exogenous feeding starts, therefore

[1]"Yolk" is the term used for the clear fluid as well as the oil globules in the yolksac.

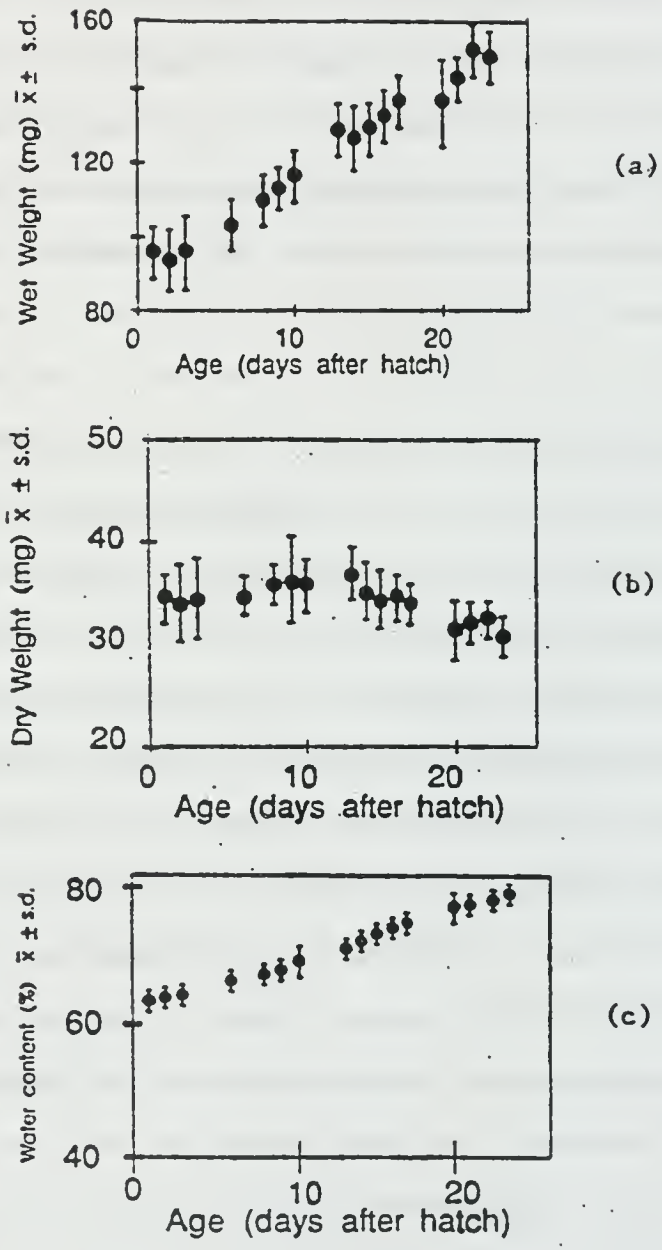

Fig. 1. Wet weight (a), dry weight (b), and % water content (c) of rainbow trout sacfry from 1d post hatch to 4d post swim-up, without feeding.

there is no overall dry weight gain until this period. Furthermore, a technique used in some longer ELS protocols, whereby growth is assessed by measuring the mean sacfry dry weight during the period from hatch to swim-up, (or mean larval dry weight in groups of separated same age larvae), may be invalid as well as inaccurate. It is complicated by the effect of any toxicant concentrations that have slightly reduced the rate of development so that although the yolksac may have been absorbed into the body cavity, there is still unabsorbed yolk within the yolksac. Before the start of exogenous feeding, this can result in swim-up fry dry weights that are slightly greater than the controls, but the greater weight does not signify increased growth.

The increase in wet weight during the development of newly hatched sacfry to non-feeding swim-up fry is normally only due to the passive absorption of ambient water induced by the absorption of yolk from the yolksac into the larva. In moderately hard water, (hardness ≈ 135 mg/L), at temperatures increasing from 7°C to 11°C, the whole fish % water content normally increases from ≈ 63 ± 0.9% to ≈ 79 ± 0.7% during this period (see Figure 1c and appendix Table A1). After exogenous feeding has been established, wet and dry weights both increase rapidly, and the ratio between them normally remains the same, therefore the whole fish % water content is stabilised. Nevertheless, in this short-term test where the length of swim-up fry exposure has to be minimised to minimise costs, the effects on growth of delays in onset and maintainence of normal exogenous feeding, with notable reductions in the amount of daily food consumption compared to the controls and other groups, are often still more readily identified by a decrease in wet weight gain than by the final dry weight alone, (see final TCP data at pH 7.4-7.7, Tables 8 and 10).

In the presence of toxicants which affect the respiratory/osmoregulatory epithelium, water absorption may be increased compared to control values at all stages of the test, causing

edema, but this would be more likely to result in acute mortality rather than sublethal chronic effects. Use of concentrations lower than incipient acute mortality in this test protocol diminishes the likelihood of this occurring. Nevertheless, edema may occur in the highest concentration group(s) with some chemicals if the highest test concentration is too close to the incipient mortality level for fry. Since edema invalidates the group mean wet weight gain as growth data, the possibility of its occurrence must be minimised unless it is known that osmoregulatory disturbances will not occur at the test concentrations and ambient water quality conditions used. The protocol (Neville, 1995) includes simple observations to be made during the swim-up stage that allows edema to be easily identified. Edema is evident if onset and maintenance of feeding is delayed by more than two days compared to the controls, and the relative amount of food consumption is noted to be severely reduced compared to lower concentration groups, yet there is no corresponding decrease in the group mean wet weight gain. On examining the individual fish at the end of the test, the whole fish will appear to be swollen, and/or the eyes may protrude. Edema will be confirmed by the shape of the dose/response growth curve which will appear to be reaching, or to have reached an asymptote in the highest concentrations, (see the DCA dose-response curves, Figures 6 and 7). In such circumstances the group mean wet weight gain must be excluded from the growth analysis.[2] Use of whole fish % water content to indicate edema is not always valid at the sacfry to early swim-up lifestage (see Discussion).

This rainbow trout early life stage growth test can be used to provide toxicity data required

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[2]Should the highest concentration exposure solution be too dark, or contain too much debris (in the case of effluents) to observe faecal casts or uneaten food, food consumption and faecal production in the controls and low to middle concentration groups will show that wet weight gains have decreased with increasing toxicant concentration in the middle concentration range even though sufficient food has been available. The maintenance of a similar wet weight gain instead of a continuing decrease in the highest toxicant concentration(s) will confirm edema.

for various approaches to the improvement of environmental quality:- 1) in the development of surface water or multimedia criteria for individual volatile and non-volatile chemicals, 2) to estimate the effects of subchronic[3] and chronic periods of exposure of larval and early juvenile coldwater fish to effluents and receiving waters, and 3) to correlate sublethal effects with tissue concentrations of individual toxicants. The latter can be used to estimate the effects of tissue concentrations found in larval and early juvenile stages of rainbow trout collected in the field.

This report describes the methodology and results of tests used during the latter stage of the test development, and the data interpretation.

[3]The term "subchronic" is used in this document for early life stage exposure periods greater than 10 days for coldwater fish, or 6 days for warmwater fish, but shorter than traditional chronic early life stage tests.

METHODOLOGY

(See "Short-term early life stage growth test using sacfry and early swim-up stages of rainbow trout (Oncorhynchus mykiss). Protocol", ISBN 0-7778-3649-1, PIBS 3359, (Neville, 1995), for detailed test procedures.)

Summary of test procedure Growth tests were carried out at the Ontario Ministry of Environment and Energy Aquatic Toxicology Laboratory in Etobicoke, Ontario, using moderately hard water (hardness ≈ 135 mg/L as $CaCO_3$). Rainbow trout late eyed-eggs were obtained from a certified disease-free trout farm (Rainbow Springs Trout Farm, Thamesford, Ontario) and reared at the laboratory for approximately two weeks prior to testing. Sacfry that hatched within an 8h or 16h period were transferred to separate rearing trays to minimise the age difference among fish used in the test. At 9-11d post hatch, (avoiding weekends), the sacfry were weighed individually, (time required ≈ 1 minute per fish - see protocol for weighing technique), then allowed a 24h recovery period before the test was initiated. During the test, the sacfry were exposed to a control and five test concentrations in a covered, static, 24 hour solution renewal system, under low light conditions (≈30 lux) at 13.5 ± 0.5° C for approximately 12 days. Twelve sacfry were used per concentration. They were exposed in individual chambers held in three replicate, covered tanks subdivided into four equal, separate, sections (see Figure 2). Toxicants used were copper sulphate, sodium dodecyl sulphate, 2,4,5-trichlorophenol[4], and 3,4-dichloroaniline[4]. Fresh test solutions were made daily from stock solutions using strongly aerated, dechlorinated Toronto tap water at 13.5 ± 0.5°C as the control and dilution water, (see Table A2).

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[4]Tests carried out at the M.O.E. laboratory with Niel Karrow, University of Waterloo. Stock chemical solutions were prepared and exposure concentrations analysed (using GC-ECD for TCP and HPLC for DCA) at the University of Waterloo.

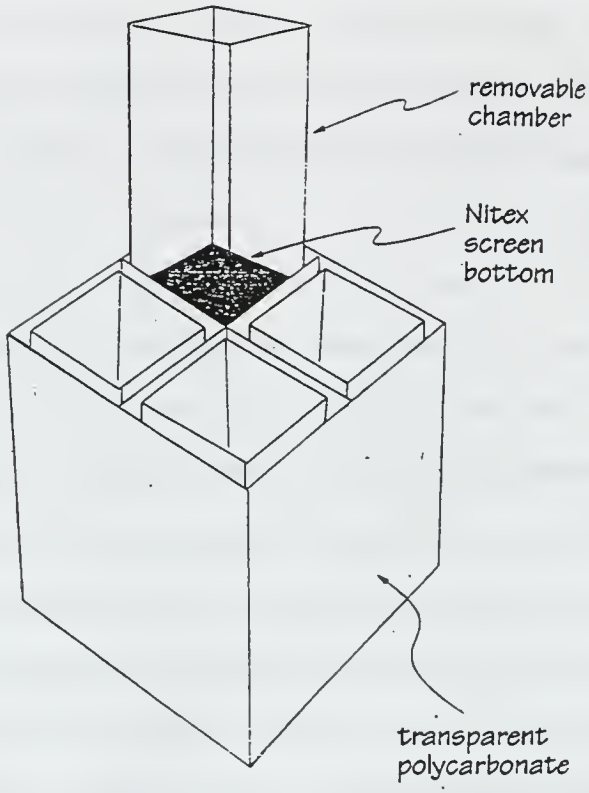

Fig. 2. Diagram of divided exposure tank with four individual exposure chambers.
(Polycarbonate cover, and 2 polycarbonate strips used for ease of handling, not shown.)

Concentrations used ranged from the low, not expected to be significantly different from the controls, to one close to, but below, the incipient acute[5] mortality level for fry. (If unknown prior to the test, this was determined by a 96h (chemicals), or estimated by a 24h (non-acutely lethal effluents) mortality test, using sacfry and swim-up fry in a static with 24h replacement test, without aeration).

Temperature, oxygen concentration, pH and conductivity were measured daily. Test concentrations were measured before and after 24h exposure on the second and penultimate

[5]Following this stage of the protocol development the highest concentration suggested for use in this growth test was changed to the incipient 12 day mortality level for rainbow trout late sacfry to feeding swim-up fry. Higher concentration ranges were used in the TCP and DCA tests to provide critical body residues high enough to prevent any gain in wet weight. These critical body residues were required for inclusion in Mr. N. Karrow's M.Sc. thesis.

days of the test, ie. once during the sacfry stage and once during feeding of the swim-up fry. Any abnormal appearance or behaviour was noted daily.

The fish were transferred to freshly prepared exposure solutions every 24h during the sacfry stage. At the swim-up fry stage, when exogenous feeding started, they were transferred twice every 24h, ie. at 18h and 24h, if the oxygen concentrations fell below 7.0 mg/L overnight due to excessive oxygen uptake by the fry and excess brine shrimp. (This was rarely necessary).

After 6d exposure, at age 17-18d when the yolk sac was mostly absorbed but still visible, exogenous feeding was initiated. The amount of food given was slightly in excess of control fish satiation. On the first day of feeding, one concentrated drop (700-1000 nauplii) of washed, 24-48h old brine shrimp (Bio-Marine Brand (Argent Platinum grade) Artemia cysts, Hawthorne, California), was given per swim-up fry, in the afternoon after solution renewal. Three feeds were given on the subsequent 4-5 days, in the early morning (after solution renewal if required), at mid-day, and again at approximately 3:00 pm after the mid-afternoon solution renewal. (Preliminary tests had shown that excess nauplii did not bioaccumulate the toxicants to a measurable extent). A simple feeding technique was used to ensure that a similar amount of food was given to all fish (see Neville, 1995). No food was given on the final day of the test. During the feeding stage, the approximate amount of food and faecal threads left in the used exposure tanks of the test groups compared to the controls was noted, to determine the relative amount of daily food consumption, and the age at which feeding began and faecal threads were first produced. (The latter are usually red and easily seen.)

At the end of the exposure period the fish were reweighed to calculate growth (as gain in wet weight), the severity of any delay in yolksac absorption was assessed by measuring the maximum width of the gap in the epithelial layer covering the yolksac (<1 mm = slight, >1 mm = moderate, >2mm = severe), and any visible abnormalities were recorded. If edema

was suspected, the shape of the dose-response curve and the amount of feeding compared to lower concentration groups was examined, and individual dry weights were measured to calculate the individual whole fish % water content. (The latter procedure has since been dropped from the routine protocol unless tissue toxicant concentrations are required). These were measured as freeze-dried weights for subsequent tissue toxicant analysis after exposure to organic chemicals.[6]

Exposure system

Changes were made to the shape of exposure vessels and the materials used in their construction during the method development, although the basic structure remained the same. Fish were exposed individually in four inner chambers contained within an outer exposure tank with a polycarbonate cover. Three tanks were used per concentration. During the copper test the inner exposure chambers were made from polymethylpentene (PMP) round jars with 1 mm drainage holes drilled into the base, divided into four sections by two interlocking plates of 1/16" thick polycarbonate. Graduated two litre borosilicate glass beakers were used as the outer exposure tanks. Problems which developed with maintaining a tight fit between the dividers and base of the inner chambers led to a change in design from round to rectangular chambers. Individual inner chambers with mesh bases were used for the fish, exposed in covered outer tanks divided into four equal sections. These were made of glass for the organic chemical tests described in this paper. They were found to be adequate but cumbersome to use, therefore in subsequent tests polycarbonate was used instead of glass. This system, shown in Figure 2, is described in detail in the protocol (Neville, 1995).

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[6]See Niel Karrow, M.Sc. thesis, 1995, University of Waterloo, Dept. of Biology, for methods of analysis of TCP exposure concentrations, TCP tissue preparation and tissue concentrations. The same methods of analysis were used for the TCP and DCA exposure concentrations reported in this paper.

Statistical Analysis

Mean individual gain in wet weight was used to calculate the significance of growth differences between the test groups and the controls, using appropriate hypothesis tests, and the LOEC and NOEC as measures of the ELS sublethal, subchronic growth response. (Linear regression analysis is not appropriate in this sublethal growth test - see Discussion). Each individually exposed fish was regarded as a replicate as there were no significant differences among the three tank replicates of each concentration group. Any group showing a severe reduction in feeding without a reduced wet weight gain compared to the next lower concentration group, (indicating edema), or a significant increase in mean % body water (showing edema), was not included in the growth analysis. (Dry weight cannot be used to determine growth in the developmental stages used in this short-term test, see Introduction; Fig.1; and Conclusions).

The Toxstat 3.3 statistical program (Gulley et al.,1991) was used for data analysis, including initial tests for normal distribution and homogeneity of variance. The data were analysed at a 5% level of significance, without transformation. "Outliers" were not eliminated unless an obvious non toxicant related abnormality was noted in that fish, (eg. injured or deformed mouth or gills), or edema had been identifed in a fish causing its "wet weight gain" to be invalid as growth data. In tests of individual chemicals, Dunnett's Procedure (or Bonferroni's T-test if any mortalities had occurred) was used. These statistical procedures also calculate the minimum significant difference from the controls, (MSD), for each test. Using the growth test method reported here, the MSD for growth reduction was 14.5 $\pm$ 4.0% of the control weight gain. The null hypothesis was always control $<$ treatment for weight data, but for whole fish % water the control and highest concentration group means were used to determine whether the null hypothesis should be control $<$ treatment, (if edema was suspected) or control $>$ treatment, (if delayed yolksac absorption was evident, see Discussion). Alternatively, Williams test could be used.

Validity of test

Variation of daily temperature and among test chamber temperatures was ≤1°C. Oxygen concentrations and pH were within normal limits and did not vary between groups unless noted. Apart from one accidental death there was no control mortality. Initial weight coefficient of variation of all individual fish used per test was ≤10%. Control fish final weight x, s.d., (n), and c.v. were 110.6 ± 18, (12), 16.3% for the copper test, (exposure temperature = 15 ± 1°C), and 64.0 ± 11.7, (47), 18.3% for the four organic chemical tests, (exposure temperature = 13.5 ± 1°C).

Preparation of Exposure solutions

a) Copper sulphate tests Fresh test solutions of copper sulphate at nominal concentrations of 0, 15, 30, 60, 90, and 120 ug Cu^{2+}/L were made daily from stock solutions using strongly aerated, dechlorinated Toronto tap water at 13.5 ± 1°C as the control and dilution water.

b) Sodium dodecyl sulphate Test solutions were prepared daily as above except that caution was taken to prevent frothing during preparation of the test concentrations and during solution renewal. (Dilution water was added slowly down the side of the volumetric flask to the appropriate volume of SDS. Mixing was obtained by gently swinging the flask in a wide circle two or three times during addition of the dilution water rather than by shaking the flask). Nominal concentrations of 0, 0.1, 0.3, 1.0, 3.0 and 10.0 mg/L were made from a stock solution prepared from an ampoule of SDS, supplied by U.S.E.P.A. as a reference toxicant.

c) 2,4,5-Trichlorophenol, (TCP)[4] A 2.0 g/L saturated stock solution of 2,4,5-trichlorophenol was prepared by adding 1.00g trichlorophenol to 500mL 1N NaOH and stirring at room temperature (20°C) for 1h. A 20 mg/L sub stock solution, made each day from the saturated stock solution, was adjusted from pH 11.5 to pH 9.5 with HCl. No carrier solution was

needed. Test solutions were prepared from the sub stock solution. Dechlorinated tap water was used for the controls and as the diluent in a series of double dilutions. The test solutions were used immediately after preparation in a 10L polycarbonate dilution tank with the dilution water at 13.5 ± 0.5°C. Nominal test concentrations were 31, 62.5, 125, 250 and 500 μg/L with pH values ranging from 7.4 to 7.7.

A preliminary test was also conducted with 2,4,5-TCP using a slightly different method of initial solution preparation. The TCP was washed with hexane before the stock solution was made, and the sub-stock solution was prepared at room temperature, then incubated at 13.5 ± 0.5°C without pH adjustment for 2-3h before use. No carrier solution was needed. The pH range was 7.7 to 8.1. Nominal test concentrations were 62.5, 125, 250, 500 and 1,000 μg/L. In preliminary tests the exposure concentrations were not measured.

d) 3,4-Dichloroaniline, (DCA)[4] A 100 mg/L stock solution was prepared in a 1L flask, heated to 75°C to melt the DCA, then stirred overnight in the dark at room temperature, before storing at 4°C. Nominal test concentrations used in a preliminary test were 21, 62.5, 125, 250 and 500 μg/L. In the subsequent test, nominal concentrations of 100, 200, 300, 400 and 500 μg/L were made by using two dilution series, a) 500 and 300 μg/L, b) 400, 200 and 100 μg/L, after appropriate dilutions of the stock solution in a 10L polycarbonate dilution tank at 13.5 ± 0.5°C. Dechlorinated tap water was used as the diluent and for the controls. No carrier solution was needed.

RESULTS

In all tests the ambient pH, oxygen saturation, free ammonia and nitrite concentrations, heavy metal concentrations apart from copper in the copper tests, and other routine water quality parameters, were within Ontario Provincial Water Quality Objectives.

a) Copper tests.

Measured copper concentrations (geometric mean of pre- and post-24h exposure concentrations) were 0, 12, 25, 48, 65, and 91 μg/L. Exposure to all copper concentrations caused significant growth reductions compared to the controls, therefore the LOEC was <12 μg/L and an NOEC was not determined, (see Table 1). The minimum significant weight difference from the controls (MSD) was 15.7%. Growth reductions ranged from 19% to 40% of control values at exposure concentrations of 12 μg/L to 65 μg/L. Toxicant induced mortalities occurred in 25% of the fish at 91 μg/L; growth reduction of the surviving fish was 65% of control growth. Whole fish % water content was not measured in this test as preliminary tests during method development had shown that no increase occurred (therefore there was no edema) in this life-stage exposed to 120 μg/L copper in moderately hard water.

There was no delay in initial feeding or faecal elimination at copper concentrations up to 65 μg/L, but both were delayed approximately 1 day at 91 μg/L. Yolksac absorption was complete in all but one of the control fish, but slightly delayed with increasing copper concentrations in most of the test groups, reaching slight to moderate delays in most fish at 91 μg/L, (see Table 2).

Whole fish copper concentrations increased from 1.4 μg/g in control fish to 2.7 μg/g in the 91 μg/L exposed group. The NOEC and LOEC of the ambient copper concentrations were 25 μg/L and 48 μg/L, respectively, (see Table 3). Copper concentrations measured in spiked tissue samples using same aged swim-up fry showed a 94% recovery, (Laboratory Services Branch,

Table 1. Growth reduction in a short-term (15d) test with the late sacfry to early swim-up life stage of rainbow trout exposed to copper in water of 135 mg/L hardness. Aug. 7 - 22, 1991.

Measured $[Cu^{2+}]$ $\mu g/L$	Initial wet weight (mg) x ± s.d. (n)	Final wet weight (mg) x ± s.d.	Gain in wet weight (mg) x ± s.d.
0	108.7 ± 7.3(12)	219.3 ± 23.2	110.6 ± 18.0
12	105.4 ± 11.0(12)	195.3 ± 22.6	89.9 ± 14.4*
25	108.5 ± 7.8(12)	201.3 ± 25.5	92.8 ± 21.0*
48	110.3 ± 10.5(12)	193.0 ± 22.4	82.7 ± 19.5*
65	102.4 ± 9.7(12)	168.3 ± 18.6	65.9 ± 14.0*
91	104.6 ± 7.5(12)	145.4 ± 21.1(9)	39.1 ± 19.2(9)*

* Significantly different from the control (Bonferroni T test, p = 0.05).
LOEC = <12 μg/L, NOEC = 0 - <12 μg/L.

The minimum significant weight difference from the controls in this larval growth test was 15.7% (17.4 mg).

Ministry of Environment and Energy, 1992).

The ambient oxygen saturation decreased by 15-20% during 24h exposure of the yolksac fry, by 30% during each of the first two days of feeding the swim-up fry, and by 40-45% at the end of each of the last 2 days of feeding, the greatest decrease being in the control groups. The pH varied between 7.0 and 7.8.

During initial stages of the protocol development, two replicates of three yolksac fry were

Table 2. Incomplete yolksac absorption in swim-up larvae after 15 d exposure to copper.

Measured $[Cu^{2+}]$ µg/L	Complete absorption	Incomplete absorption	
		Maximum width of exposed yolksac surface	
		≤ 1mm	> 1mm
0	92%	8%	0
12	83%	17%	0
25	92%	8%	0
48	50%	50%	0
65	67%	33%	0
91	20%	50%	30%

Table 3. Copper concentrations in whole fish tissue after 15d exposure.

Measured $[Cu^{2+}]$ µg/L	Wet weight of tissue analyzed (g) x ± s.d. (n)	Whole fish $[Cu^{2+}]$ µg/g x ± s.d.
0	0.818 ± 0.021 (3)	1.4 ± 0.2
12	0.682 ± 0.060 (3)	1.7 ± 0.2
25	0.730 ± 0.023 (3)	1.8 ± 0.2
48	0.740 ± 0.088 (3)	2.2 ± 0.3*
65	0.592 ± 0.037 (3)	2.2 ± 0.3*
91	0.351 ± 0.096 (3)	2.7 ± 0.6*

* Significantly different from controls. (Dunnett's test, p = 0.05).
The minimum significant difference in this test was 0.7 µg/g, (51.3% of control).

Table 4 Growth of rainbow trout fry exposed to copper in water of 10 mg/L hardness for 10d. May 26 - June 5, 1989.

Nominal $[Cu^{2+}]\mu g/L$	Initial wet weight (mg) $\bar{x} \pm$ s.d.(n)	Final wet weight (mg) $\bar{x} \pm$ s.d.(n)	Gain in wet weight $\bar{x} \pm$ s.d.(n)	Mortality
0	91.5 ± 2.6 (6)	117.5 ± 13.8(6)	26.0 ± 11.5(6)	0
3	89.4 ± 7.0 (6)	108.4 ± 15.3(5)	19.0 ± 10.3(5)	17%
10	83.3 ± 9.1 (6)	89.6(1)	6.3(1)	83%
30	86.6 ± 6.7 (6)	0	0	100%

exposed to copper concentrations ranging from 3 to 30 μg/L in very soft water for 10 days, after being cultured in water with hardness gradually decreasing to ≈ 10mg/L as $CaCO_3$. Exposure solutions were renewed every 24h. At swim-up they were fed with starter trout chow for two days. Growth was approximately half that of controls in two fish at 3 μg/L and one mortality occurred. However, with the small number of replicates and fish these effects were not statistically significant. A nominal concentration of 10 μg/L caused 83% mortality and minimal growth in the surviving fish. All fish died at 30 μg/L (see Table 4).

b) Sodium dodecyl sulphate (SDS) test.

Yolksac fry were also exposed to sodium dodecyl sulphate in moderately hard water during the initial tests. Reduced growth occurred at nominal concentrations of 0.3 mg/L and 1.0mg/L and a loss of weight occurred in the one surviving fish at 3.0 mg/L. At 10 mg/L, all fish died within 24 hours. Only three fish per group were available for this test and there were no replicate groups therefore analysis for significant differences between the control and test groups was not possible (see Table 5).

Table 5. Growth of rainbow trout fry exposed to **sodium dodecyl sulphate** in water of 135 mg/L hardness for 10d. Nov.21 - Dec.1,1988

Nominal [SDS] mg/L	Initial wet weight (mg) $\bar{x} \pm$ s.d.(n)	Final wet weight (mg) $\bar{x} \pm$ s.d.	Gain in wet weight (mg) $\bar{x}$	Mortality
0	121.6 ± 19.3(3)	141.2 ± 22.6	19.6	0
0.1	120.1 ± 26.3(3)	140.7 ± 35.1	20.6	0
0.3	122.6 ± 11.2(3)	136.3 ± 21.9	13.8	0
1.0	129.3 ± 10.5(3)	127.4 ± 21.1	-1.9	0
3.0	121.9 ± 21.1(3)	94.0	-27.9	66%
10.0	115.7 ± 28.0(3)	0	0	100%

c) Preliminary TCP test In this test the pH range was 7.7 - 8.1. All fish in group 6, exposed to 1,000 µg/L 2,4,5-TCP, died during the first 2-3 days of exposure. There were no mortalities at 0 - 500 µg/L, (nominal concentrations). Growth was reduced by 21.6% (14.0 mg) at the LOEC (125 µg/L), and 3.9% (2.5 mg) at the NOEC (62.5 µ/L). The minimum significant weight difference was 12.8%, (8.3 mg), (see Table 6). Freeze dried weights were also significantly reduced at 125 - 500µg/L, (groups 3 - 5), but the weight was slightly greater at 250 µg/L than at 125 µg/L, (Table 7). There were no significant differences in % body water. Yolksac absorption was not recorded in this test. There was no delay in onset of feeding or faecal production in any group, but the quantity of both were slightly reduced at 500 µg/L. Ambient oxygen concentrations were slightly lower than the controls during the first 5d of the sacfry stage after exposure to 250 and 500 µg/L, but were never below 7.0 mg/L or 6.0 mg/L, (67% or 57% saturation), respectively.

Table 6. Growth reduction in a preliminary short-term test with the late sacfry to early swim-up life stage of rainbow trout exposed to **2,4,5-trichlorophenol** in water of 135 mg/L hardness for 12d. **(pH range = 7.7 - 8.1)** June 10 -22, 1992

Nominal [TCP] µg/L	Initial wet weight (mg) x ± s.d.(n)	Final wet weight (mg) x ± s.d.	Gain in wet weight (mg) x ± s.d.
0	112.0 ± 6.2(12)	176.8 ± 13.0	64.7 ± 10.7
62.5	112.4 ± 6.6(12)	173.8 ± 11.0	62.2 ± 7.9
125	112.2 ± 4.6(12)	162.9 ± 12.7	50.7 ± 11.3*
250	112.7 ± 6.2(12)	160.0 ± 7.8	47.3 ± 7.4*
500	112.3 ± 4.0(12)	142.8 ± 8.5	30.4 ± 7.6*

* Significantly different from the control (Dunnett's test, p = 0.05).
LOEC = 125 µg/L, NOEC = 62.5 µg/L

The minimum significant weight difference from the controls in this larval growth test was 12.8% (8.3 mg).

Table 7. Freeze-dried weights and whole fish % water content in rainbow trout fry after a preliminary short-term test with the late sacfry to early swim-up life stage exposed to **2,4,5-trichlorophenol** in water of 135 mg/L hardness for 12 days. **(pH range = 7.7 - 8.1)**

Nominal [TCP] µg/L	Freeze dried weight (mg) x ± s.d.(n)	Whole fish % water x ± s.d.
0	29.5 ± 1.7(12)	83.3 ± 0.5
62.5	29.1 ± 1.8(12)	83.3 ± 0.4
125	26.9 ± 2.5(12)*	83.5 ± 0.7
250	27.2 ± 1.2(12)*	83.0 ± 0.6
500	23.7 ± 1.5(12)*	83.4 ± 0.5

* Significantly different from the control, (Dunnett's test, p = 0.05). LOEC = 125 µg/L, NOEC = 62.5 µg/L (Nominal concentrations.)

The minimum significant freeze-dried weight difference in this test was 5.5% of the control, (1.6 mg).

There was no significant difference in whole fish % water content between the control group and any test group.

The minimum significant whole fish % water difference in this test was 0.6% of the control, (0.5% water).

d) Final 2,4,5-trichlorophenol test.

Measured TCP concentrations were 0, 34 ± 14, 58 ± 13, 107 ± 18, 211 ± 31, and 438 ± 54 µg/L, (Karrow,1995), (see Table 8). The pH ranged from 7.4 to 7.7. One accidental mortality occurred in each of groups 1 and 2, and, in contrast to the preliminary test at pH 7.7 - 8.1, described above, one toxicant induced mortality occurred in group 6 at the nominal concentration of 500 µg/L TCP, (measured concentration = 438 µg/L). Furthermore, exposure to all TCP concentrations caused significant reductions in growth compared to the controls, therefore the LOEC was <34 µg/L. The minimum significant weight difference was 20.1%,

Table 8. Growth reduction in the final short-term test with the late sacfry to early swim-up life stage of rainbow trout exposed to **2,4,5-trichlorophenol** in water of 135 mg/L hardness for 12d. (pH range = 7.4 - 7.7) July 26 - Aug. 7, 1992.

Measured [TCP] µg/L	Initial wet weight (mg) x ± s.d.(n)	Final wet weight (mg) x ± s.d.(n)	Gain in wet weight (mg) x ± s.d.(n)
0	122.4 ± 5.8(12)	182.1 ± 16.4(11)▲	59.7 ± 16.1(11)
34	120.2 ± 6.1(12)	164.9 ± 14.9(11)▲	44.8 ± 12.2(11)*
58	121.8 ± 6.4(12)	161.9 ± 19.0(12)	40.1 ± 15.0(12)*
107	121.1 ± 7.4(12)	164.5 ± 14.8(12)	43.5 ± 10.0(12)*
211	119.6 ± 3.9(12)	155.8 ± 8.3(12)	36.2 ± 7.1(12)*
438	121.7 ± 5.4(12)	116.8 ± 10.1(11)	-7.2 ± 5.2(10)*#

▲ One accidental mortality occurred in this group.

One edematous fish was excluded from this group.

* Significantly different from the control (Bonferroni T-test, p = 0.05).
LOEC = <34 µg/L, NOEC = 0 - <34 µg/L

The minimum significant weight reduction compared to the controls in this larval growth test was 20.1% (12.0 mg).

(12.0 mg). At the LOEC, growth was reduced by 25%, (14.9 mg). As this was the lowest exposure concentration the NOEC was not determined and at present can only be reported as <34 μg/L. There was no delay in onset of feeding or faecal elimination at TCP concentrations up to 107 μg/L, but both were delayed by one day at 211 μg/L, (group 5). Fish exposed to 438 μg/L TCP appeared not to be feeding, and there was no faecal production. Subsequently, in this group, by the end of the 12 day test there was a loss in wet weight instead of a gain. Yolksac absorption into the body cavity was complete in all groups except at 438 μg/L. At this concentration, it was incomplete in all of the fish, and was severely delayed in up to 73% of the fish (see Table 9). One fish in this group was also visibly edematous, therefore its wet weight gain was excluded from the group growth data. Freeze dried weights were reduced in all groups compared to the controls, but the reductions were not statistically significant in groups 2 and 4, exposed to 34 and 107 μg/L, respectively, (see Table 10). At 438 μg/L, the mean % body water content was significantly decreased. Throughout the test there was little

Table 9. Incomplete yolksac absorption in swim-up larvae after 12d exposure to 2,4,5-trichlorophenol at pH 7.4-7.7. (Final TCP test)

Measured [TCP] μg/L	Complete absorption	Incomplete absorption	
		Maximum width of exposed yolksac surface	
		$\leq$ 1mm	$>$ 1mm [1]
0	100%	0	0
34	100%	0	0
58	92%	8%	0
107	100%	0	0
211	100%	0	0
438	0	27%	73%

[1] Differentiation between $>$1mm and $>$2mm was not made at this stage of the test development.

activity in the fish in this group, and many were very dark in colour. (For whole fish TCP concentrations, see Karrow, 1995).

In the sacfry stage, ambient oxygen saturations decreased in all groups to approximately 70% in 24h during the first two days of exposure, but were then consistently lower at 438 μg/L, (73-60%), than in all other groups, (86-78%). This pattern was still evident until the last three days of the test when it ranged from 83-75% in all groups.

Table 10. Freeze-dried weights and whole fish % water content in rainbow trout fry after the final short-term test with the late sacfry to early swim-up life stage exposed to 2,4,5-trichlorophenol in water of 135 mg/L hardness for 12 d. (pH range = 7.4 - 7.7)

Measured [TCP] μg/L	Freeze dried weight (mg) $\bar{x} \pm$ s.d.(n)	Whole fish % water $\bar{x} \pm$ s.d.
0	29.8 ± 3.3(11)▲	83.6 ± 0.5
34	27.3 ± 2.9(11)▲	83.4 ± 0.4
58	26.6 ± 3.7(12)*	83.6 ± 0.4
107	27.7 ± 3.1(12)	83.2 ± 0.4
211	25.9 ± 2.1(12)*	83.4 ± 0.6
438	21.8 ± 3.0(11)*	81.3 ± 2.5#

▲ One accidental mortality occurred in this group.

* Significantly different from the control, (Bonferroni T test, p = 0.05). LOEC = 58 μg/L, NOEC = 34 μg/L and 107 μg/L

The minimum significant freeze-dried weight difference from the controls in this larval growth test was 10.2% (3.0 mg).

\# Significantly different from the control, (Bonferroni T test, p = 0.05). LOEC = 438 μg/L, NOEC = 211 μg/L

The minimum significant whole fish % water difference from the controls in this larval growth test was 1.3% (1.1% water).

(e) Preliminary DCA test

The nominal concentrations of the LOEC and NOEC were 250 μg/L (group 5) and 125 μg/L (group 4); growth was reduced by 35.6% (22.3 mg) and 7.8% (4.9 mg), respectively. The minimum significant growth reduction was 16.7% (10.4 mg). There was a non-linear response between the two highest concentration groups, since the decrease in wet weight gain between groups 5 and 6 was only half of that between groups 4 and 5, (see Table 11 and Figure 6). In group 6, exposed to 500 μg/L, the calculated mean whole fish % water content was

Table 11. Growth reduction in a preliminary short-term test with the late sacfry to early swim-up life stage of rainbow trout exposed to **3,4-dichloroaniline** in water of 135 mg/L hardness for 12d. Dec. 11 - 23, 1992.

Nominal [DCA] μg/L	Initial wet weight (mg) x ± s.d.(n)	Final wet weight (mg) x ± s.d.	Gain in wet weight (mg) x ± s.d.
0	113.7 ± 4.2(12)	176.3 ± 8.4	62.6 ± 7.7
21	115.0 ± 6.2(12)	179.0 ± 9.2	64.1 ± 4.9
63	115.5 ± 6.6(12)	176.5 ± 11.8	61.0 ± 9.0
125	113.9 ± 1.9(12)	171.6 ± 8.5	57.7 ± 8.9
250	116.6 ± 4.6(12)	156.6 ± 8.3	40.3 ± 8.2(11)*
500	114.0 ± 5.7(12)	146.4 ± 20.6(11)	32.5 ± 19.1(11)**

** There was a significant increase in % body water content (Williams test) signifying edema in this group, therefore the group was not included in the Bonferroni T test for growth analysis.

* Significantly different from the control (Bonferroni T test, p = 0.05).
LOEC = 250 μg/L, NOEC = 125 μg/L (nominal concentrations).
One edematous fish was excluded from the mean wet weight gain for this group.

The minimum significant weight difference from the controls in this larval growth test was 16.7% (10.4 mg).

significantly increased (see Table 12). One toxicant induced mortality occurred on the last day of the test. The surviving fish were all exophthalmic, ten fish had haemorrhagic areas in the eyes, and/or yolksac or epithelium, and in three fish the whole body was also visibly swollen. Yolksac absorption was severely delayed in 8 of the fish (see Table 13). These effects were less severe in group 5. Only one fish resembled those in group 6, less severe haemorrhage or swelling occurred in three others, and yolksac absorption was slightly to moderately delayed in ten fish. The wet weight gain of the one severely exophthalmic, swollen, and haemorrhagic fish in this group was twice that of the group mean and therefore was excluded from the

Table 12. Whole fish freeze-dried weights and % water in rainbow trout fry after a preliminary short-term test with the late sacfry to early swim-up life stage exposed to **3,4-dichloroaniline** in water of 135 mg/L hardness for 12d.

Nominal [DCA] µg/L	Freeze dried weight (mg) x ± s.d.(n)	Whole fish % water x ± s.d.
0	29.6 ± 1.2(12)	83.2 ± 0.3
21	30.2 ± 1.4(12)	83.1 ± 0.5
63	30.3 ± 1.9(12)	82.8 ± 0.4
125	28.4 ± 2.2(12)	83.4 ± 1.0
250	27.3 ± 2.1(12)*	83.0 ± 1.2
500	22.7 ± 1.6(11)*	84.2 ± 2.2(11)#

* Significantly different from the control, (Bonferroni T test, p = 0.05).
LOEC = 250 µg/L, NOEC = 125 µg/L (nominal concentrations)

The minimum significant whole fish freeze-dried weight difference in this larval growth test was 5.9% of the control, (1.8 mg).

\# Significantly different from the control, (Williams' test, p = 0.05).
LOEC = 500 µg/L, NOEC = 250 µg/L (nominal concentrations).
(The MSD of the Bonferroni T test was 1.3% of the control (1.1% water).)

growth analysis. Two other fish appeared slightly to moderately swollen but their wet weight gains were within one standard deviation of the group mean and were not excluded. In group 4, 50% of the fish had a slightly pink tinge in the epithelium, and yolksac absorption was slightly or moderately delayed in seven fish, but no other abnormalities were seen and growth was not significantly reduced.

Table 13. Incomplete yolksac absorption in swim-up larvae after 12d exposure to **3,4-dichloroaniline** (preliminary test).

Nominal [DCA] µg/L	Complete absorption	Incomplete absorbtion	
		Maximum width of exposed yolksac surface	
		≤ 1mm	> 1mm
0	92%[1]	8%	0
21	83%[1]	17%	0
63	83%[1]	17%	0
125	42%[1]	50%	8%
250	17%[1]	66%	17%
500	0	18%	82%[2]

[1] Most of these fish showed a "hairline slit" in the overlying epithelium, but the yolksac was completely absorbed into the body cavity.

[2] In this group 75% of the fish had a maximum width of exposed yolksac ≥ 2mm.

There was no evidence of feeding in any of the swim-up fry in group 6. Food consumption and faecal production were severely reduced in the group 5 fish, and most did not feed after the fourth day; they were also slightly reduced in group 4 and in a few of the group 3 fish by the fourth day of feeding.

Oxygen concentrations were ≥70% saturation in all groups throughout the test. However, the amount of oxygen consumed in groups 5 and 6 was reduced compared to the lower concentration groups by the final day. The pH ranged from 7.4 to 7.8. The temperature in this test was 12.5 ± 0.5 °C.

(f) Final 3,4-DCA exposed fish.

Measured DCA concentrations were 0, 55 ± 37, 139 ± 29, 199 ± 44, 326 ± 55, and 448 ± 86 µg/L. There were no mortalities. Growth reduction in the LOEC (139 µg/L, group 3), and NOEC (55 µg/L, group 2), was 21.7% (14.9 mg) and 2.8% (1.9 mg), respectively, (see Table 14). The minimum significant weight difference was 11.3%, (7.8 mg). In group 6,

Table 14. Growth reduction in the final short-term test with the late sacfry to early swim-up life stage of rainbow trout exposed to 3,4-dichloroaniline in water of 135 mg/L hardness for 12d. June 3 - 15, 1993.

Measured [DCA] µg/L	Initial wet weight (mg) x ± s.d.(n)	Final wet weight (mg) x ± s.d.	Gain in wet weight (mg) x ± s.d.
0	111.2 ± 6.7(12)	179.9 ± 12.6	68.7 ± 10.2
55	108.7 ± 6.3(12)	175.5 ± 9.9	66.8 ± 5.2
139	110.6 ± 5.4(12)	164.5 ± 10.2	53.9 ± 7.6*
199	111.6 ± 6.1(12)	147.1 ± 13.4	35.5 ± 11.5*
326	109.1 ± 6.7(12)	142.0 ± 13.5	30.2 ± 8.4(10)^#
448	110.8 ± 5.6(12)	144.3 ± 13.1	33.5 ± 11.5**#

** There was a significant increase in mean % body water content (signifying edema) in this group, therefore the group was not included in the Dunnett's test for growth analysis.

^ Two edematous fish were excluded from this group mean wet weight gain.

Tukey's test showed no significant differences among wet weight gains of groups 4, 5, and 6, therefore group 5 as well as group 6 was not included in the Dunnett's test for growth analysis.

* Significantly different from the control (Dunnett's test, p = 0.05).
LOEC = 139 µg/L, NOEC = 55 µg/L

The minimum significant weight difference from the controls in this larval growth test was 11.3% (7.8 mg).

exposed to 448 μg/L, there was a significant increase in % body water, indicating edema (see Table 15) and yolksac absorption was severely delayed in 91% of the fish, (Table 16). The mean wet weight gain was slightly greater than in group 5, exposed to 328 μg/L, and similar to that in group 4, exposed to 199 μg/L. Although growth was still significantly reduced, the group 6 data were not included in the statistical growth analysis so that the increase in wet

Table 15. Whole fish freeze-dried weights and % water in rainbow trout fry after the final short-term test with the late sacfry to early swim-up life stage exposed to 3,4-dichloroaniline in water of 135 mg/L hardness for 12d.

Measured [DCA] μg/L	Freeze dried weight (mg) x ± s.d.(n)	Whole fish % water x ± s.d.
0	30.5 ± 2.5(12)	83.1 ± 0.4
55	29.1 ± 1.9(12)	83.5 ± 0.4
139	28.2 ± 2.0(12)*	82.9 ± 0.3
199	25.5 ± 2.4(12)*	82.7 ± 0.6
326	23.3 ± 1.5(12)*	83.6 ± 1.0
448	23.2 ± 2.5(12)*	84.1 ± 1.1#

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* Significantly different from the control, (Dunnett's test, p = 0.05).
LOEC = 139 μg/L, NOEC = 55 μg/L

The minimum significant whole fish freeze-dried weight difference in this larval growth test was 6.7% of the control, (2.0 mg).

\# Significantly different from the control, (Dunnett's test, p = 0.05).
LOEC = 448 μg/L, NOEC = 326 μg/L

The minimum significant whole fish % water difference in this larval growth test was 0.8% of the control, (0.7% water).

weight due to edema, rather than growth, would not affect the test sensitivity. In group 5, yolksac absorption was severely delayed in 50% of the fish, and in two fish the % body water was as severely increased as in group 6, therefore their wet weight gains were not included in the group mean for growth analysis. Nevertheless, the mean wet weight gain was still similar to that in groups 4 and 6, (see Table 14 and Figure 7), therefore group 5 was also excluded from the growth analysis. The group 4 fish were all dark, indicating stress, but they showed no signs of edema. Freeze dried weights were significantly reduced in groups 3-6, but there was no reduction between groups 5 and 6, (see Table 15).

Some delay in onset of feeding and faecal production was noted in all groups compared to the controls, increasing from <1d in group 2 to 1d in groups 3 and 4, and 2-6d in groups 5 and

Table 16. Incomplete yolksac absorption in swim-up larvae after 12d exposure to 3,4-dichloroaniline. (Final DCA test)

Measured [DCA] µg/L	Complete absorption	Incomplete absorption	
		Maximum width of exposed yolksac surface	
		≤ 1mm	> 1mm
0	-[1]	-[1]	0
55	-	-	0
139	-	-	17%
199	-	-	25%
326	-	-	50%
448	-	-	91%[2]

[1] Differentiation between complete absorption and exposed yolksac surface < 1mm in width was not noted in this test.

[2] In this group 42% of the fish had a maximum width of exposed yolksac ≥ 2mm

6. Feeding quantity was also noted to be slightly reduced in group 4 and severely reduced in groups 5 and 6. In the latter many of the fish appeared not to be eating, or may have fed minimally for 1-2d but not digested the food as there was no faecal production. There was little activity in the group 6 fish during the last 2d of the test. Many of the fish were noted to have swollen, unabsorbed yolksacs as well as swollen body tissues. Of these fish, and also in the group 5 fish, 40% were light in colour, 60% were dark, therefore, unlike the effects of mildly toxic conditions, in edematous fish there was no clear correlation between depth of colour and severity of toxic effects.

Ambient oxygen concentrations after 24h exposure on the last two days of the test were 7.5 - 8.2 mg/L in most groups, but were higher in group 6, (8.2 - 8.7 mg/L), indicating reduced activity. The pH ranged from 7.4 to 7.7.

DISCUSSION

Interpretation of the copper and TCP test results.

Wet weight gain data from the copper and TCP tests show a small, non-linear growth response in the lower test concentrations, (see Figures 3 and 5), though, unlike the hormetic growth response discussed by Stebbing, (1982), this non-linear growth response is small and rarely exceeds that of the controls. The results indicate that at concentrations close to the LOEC, minor behavioural and/or physiological responses to small, gradually developing, metabolic changes in the fish throughout the subchronic exposure period can cause fluctuations in the dose/growth response curve, even though the behavioural or physiological responses are too small to be observed. At the LOEC, the fish may not respond to any adverse effect of the chemical, but a slightly higher concentration may stimulate a response in some of the fish. If these fish respond with a slight, not visibly detectable, decrease in activity throughout the exposure period, the energy conserved could be used for growth, resulting in a slightly higher mean gain in weight than the LOEC exposed fish. This is therefore a homeostatic response. At the next higher concentration, reducing activity may no longer compensate for the adverse metabolic effect, therefore energy available for growth would again be slightly reduced. In this group of fish the weight gain may be similar to, or only slightly less than, the LOEC group. Thus, the first few concentrations may cause similar differences in growth reduction compared to the controls, (see the copper data, groups 2, 3, and 4 in Table 1 (p.17) and Figure 3; and the TCP data at pH 7.4-7.7, groups 3, 4, and 5 in Tables 8 (p.23) and 10 (p.25), and Figure 5). This data should not be transformed when the non-linearity is due to a biological response to the toxicant. However, it does make the use of hypothesis tests for statistical analysis of the growth response at this lifestage more accurate than regression analysis or linear interpolation.

Validation of the copper and TCP wet weight data.

Copper. Dry weights and % body water content were not measured in the copper test described here as earlier copper tests had shown that edema did not occur under the exposure conditions used. In this test, none of the fish appeared to be swollen and observations on food consumption and faecal elimination showed these to be delayed only in group 6, by only one day. Thus, all of the wet weight data were valid. In the lower concentration groups the non-linear response described above was evident in groups 2, 3 and 4, (see Figure 3), due to a homeostatic response in group 3, but this did not affect data interpretation. However, the lowest concentration used was not quite low enough to determine the NOEC, therefore the LOEC was reported as <12 µg/L. Nevertheless, the non-linear response indicated that the LOEC was likely to be very close to 12 µg/L.

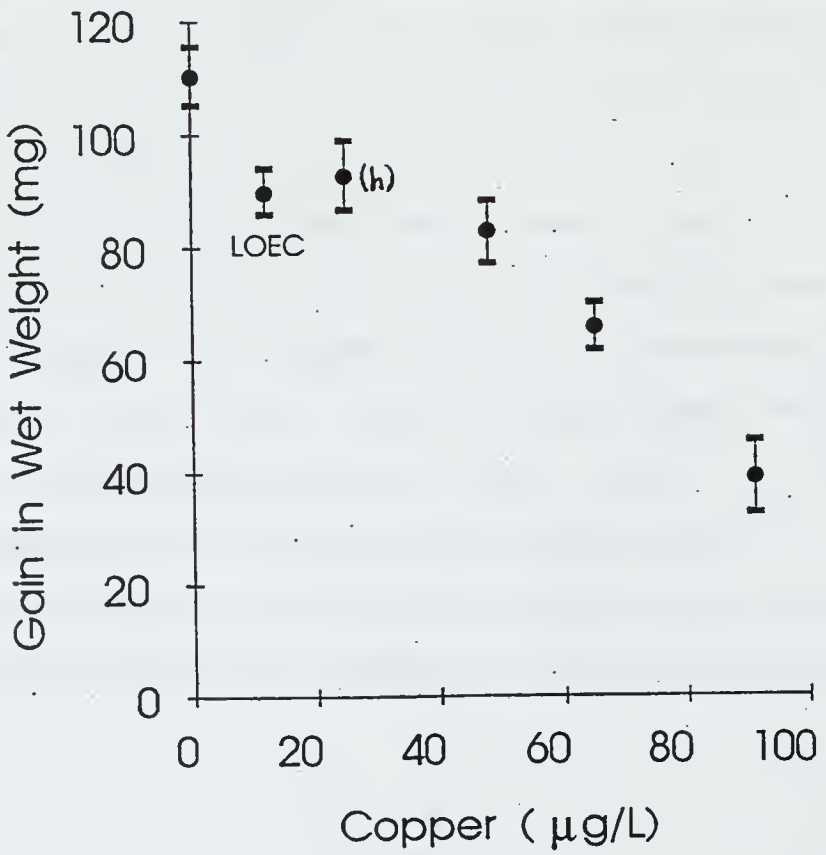

Fig. 3. Dose/response curve (mean ± s.e.) showing wet weight gain of rainbow trout late sacfry to fed swim-up fry exposed to copper for 15 days. (h) = homeostatic response

Preliminary 2,4,5-TCP The toxicity of 2,4,5-TCP is known to decrease with increasing pH, as the concentration of the phenate ion decreases (Saarikoski and Viluksela, 1981). This is clearly shown by the results of this test at pH 7.7 - 8.1 (see Figure 4) compared to those at pH 7.4 - 7.7 (see Figure 5), and demonstrates the sensitivity of this growth test. Feeding was not delayed in any group in this test, although the amount of food consumed was noted to be slightly reduced at 500 µg/L TCP. No evidence of edema was noted in any fish at the end of the test. The wet weight gains in all test groups were therefore valid, and identified 125 µg/L (group 3) as the LOEC and 62.5 µg/L as the NOEC (nominal concentrations).

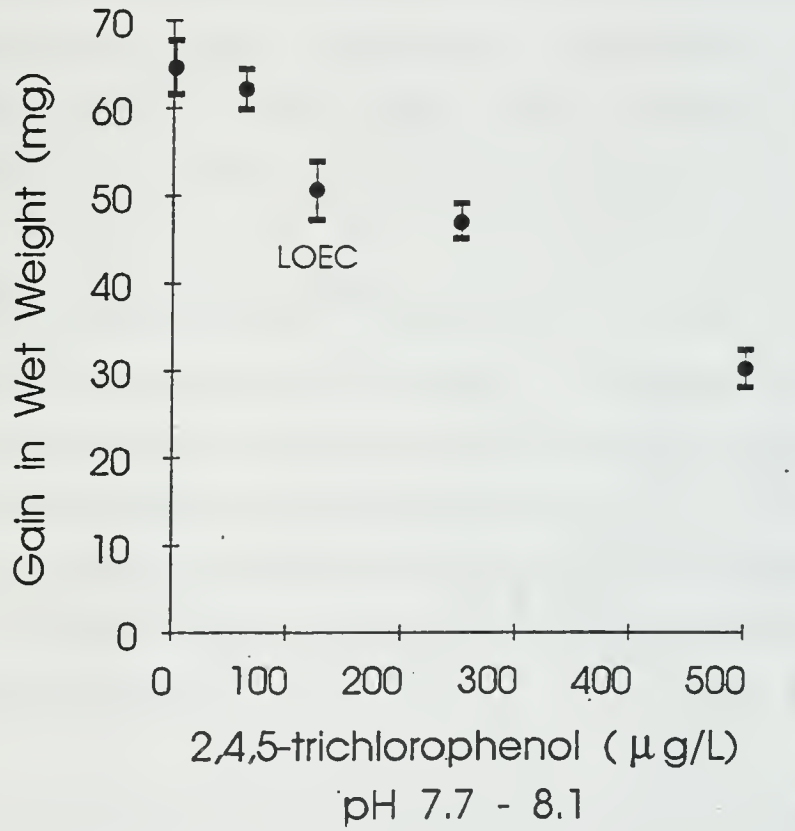

Fig. 4. Dose/response curve (mean ± s.e.) showing wet weight gain of rainbow trout late sacfry to fed swim-up fry exposed to 2,4,5-TCP in a preliminary test at pH 7.7-8.1 for 12 days.

Final 2,4,5-TCP In the test at pH 7.4-7.7, there was no evidence of edema in group 5 and the lower concentration groups, therefore the wet weight gains in these groups were valid (Figure 5). After exclusion of the one visibly severely swollen fish in group 6, exposed to 438 µg/L TCP, the lack of feeding and subsequent loss of weight, the absence of visible signs of swelling, and the decrease in whole fish % water content due to the severely delayed yolksac

absorption of the fish, showed that these wet weights were also valid growth data. As in the copper test, the non-linear homeostatic response evident in the lower concentration groups did not cause problems in interpretation of the wet weight gain data though, again, the concentrations were not quite low enough to determine an NOEC. The LOEC was therefore reported as <34 μg/L.

The greater sensitivity of the wet weight gain compared to the dry weight for growth analysis in swim-up fry, even after 5 days of exogenous feeding, is evident in this test (see Tables 8 (p.23) and 10 (p.25)). Although both data sets showed a non-linear response in groups 3, 4, and 5, the dry weights in group 4 as well as group 2 were not significantly different from the controls (see Table 10 (p.25)). The LOEC and NOEC for dry weight were either 58 μg/L (group 3) and 34 μg/L (group 2) respectively, or 211 μg/L (group 5) and 107 μg/L (group 4) respectively. In either case, the "NOEC"s for dry weight caused significant decreases in wet weight gain.

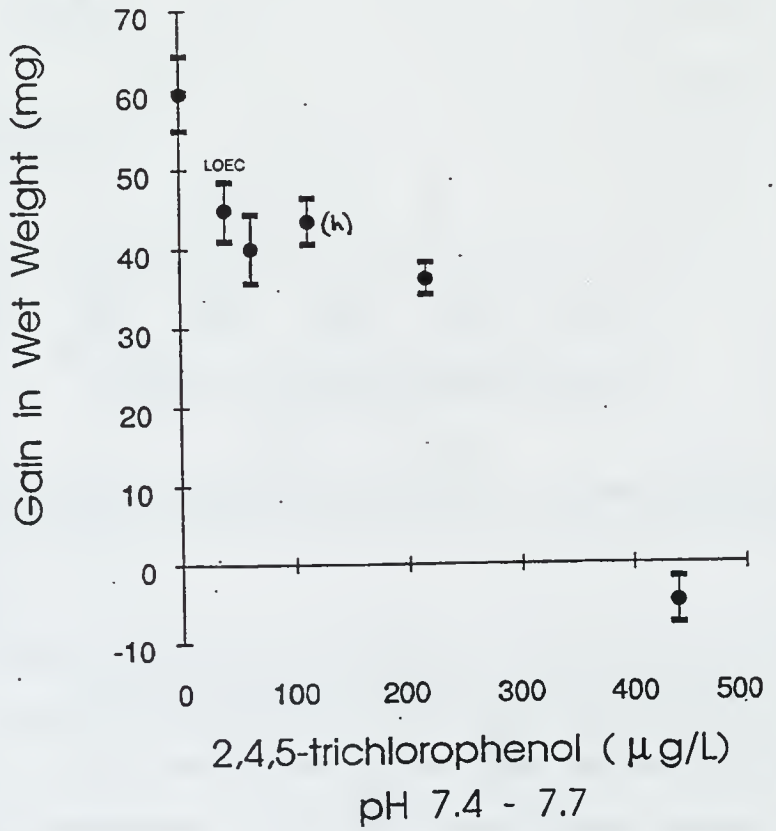

Fig. 5. Dose/response curve (mean ± s.e.) showing wet weight gain of rainbow trout late sacfry to fed swim-up fry exposed to 2,4,5-TCP in the final test at pH 7.4-7.7 for 12 days.
(h) = homeostatic response

Interpretation of the DCA tests. Interpretation of the data from both DCA tests was difficult as the concentration ranges used were higher than those required for the growth test. They were designed to reach incipient mortality levels in order to measure critical tissue DCA concentrations. The interpretation problems encountered are avoided in the normal growth test protocol by using a preliminary range finding test. Concentrations causing any swelling of the body tissues, severe protrusion of the eyes, severe tissue haemorrhage, or severely delayed yolksac absorption are too high to be used in the growth test. Concentrations causing severely reduced feeding compared to the next lowest toxicant concentration should also show a severe reduction in wet weight gain. In the preliminary DCA test, edema was indicated by the smaller decrease between the wet weight gains of groups 5 and 6 than between groups 4 and 5, despite the larger increase in DCA concentration (see Figure 6, p.39).

Edema can usually easily be confirmed in doubtful cases if the test protocol has been expanded to include dry weight measurements, normally not required unless the test is to be used to correlate tissue toxicant concentrations with the sublethal effects. By measuring individual swim-up fry dry weights in all groups at the end of the test as well as wet weights, the individual ratio of wet to dry weights can be calculated to determine the whole fish % water content. A significant increase in % water content confirms edema, and invalidates the wet weight gain as growth data.

A significantly reduced % water content calculated at the end of this test indicates delayed yolk absorption with no development of exogenous feeding, effects that also may be found in fish exposed to concentrations close to incipient mortality levels, (see the TCP at pH 7.4-7.7 group 6 data, Tables 10 (p.25) and 11 (p.26)). These effects should have already been noted during the test. In this case, the wet weight gain data are still valid.

Difficulty arises when edema and delayed yolksac absorption both occur, since the mean % water content may appear to be normal, thus confusing rather than clarifying data

interpretation (see DCA data, Group 5, Tables 14 (p.29), 15 (p.30) and 16 (p.31)). However, in severely affected groups or individual fish within a moderately affected group, the combined visible effects can still be easily identified. Swollen body tissues and/or exophthalmia in conjunction with delayed yolksac absorption will be noted as well as the delayed onset and severely reduced quantity of feeding. If the effects are less severe, delayed yolksac absorption and lack of exogenous feeding will still be obvious but visible identification of the edema may be more difficult. In either case, the flattened dose/response growth curve will identify the presence of edema as well as the more obvious delayed yolksac absorption in the affected group. Hence, due to the edema, the group mean wet weight gain is invalid as growth data.

When significant osmoregulatory dysfunction does occur in this growth test, causing severe edema, this must be reported. The mean wet weight gain of the affected group must not be included in the growth analysis as the gain is not all related to growth. As with all toxicological effects at sublethal concentrations, the weaker individuals in a group of fish will show a deleterious effect before the stronger fish, therefore in the next highest concentration group a few fish may also be edematous. This group need not be excluded from the growth analysis if there is no indication of concomitant severely delayed yolksac absorption, and the mean % body water content is not significantly higher than the controls. However, individual fish within this group with a wet weight gain exceeding the group mean plus two standard deviations should be excluded.

Validation of the DCA wet weight data.

Preliminary 3,4-DCA The severe exophthalmia, swollen body tissues and haemorrhagic areas noted in the highest concentration group were all clear evidence of edema and were sufficient to invalidate the group 6 wet weight gains as growth data. These observations were substantiated by the much smaller decline in mean wet weight gains between groups 5 and

6 than between groups 4 and 5 (see Figure 6) despite the complete absence of exogenous feeding in group 6. However, the delay in yolksac absorption in group 6 (see Table 12 (p.27)) was so severe that the mean whole fish % water content did not reflect the extent of the edema, and was only shown to be significantly increased by Williams test, not by the Bonferroni T test. In group 5, the wet weight gain and % water content of the one fish resembling the most severely affected group 6 fish were greater than the group mean plus two standard deviations and were discarded. Its wet weight gain, alone, had prevented the variance of all the group mean wet weight gains from being homogeneous. Two other fish in this group noted to be slightly swollen at the end of the test were not discarded since absorption of their yolksacs was not excessively delayed and their data were within one standard deviation of the group means. Exclusion of the one severely edematous fish reduced the group mean wet weight gain by 10% (4.0 mg); exclusion of the two slightly swollen fish would have further reduced it by only 2.7% (1.1 mg).

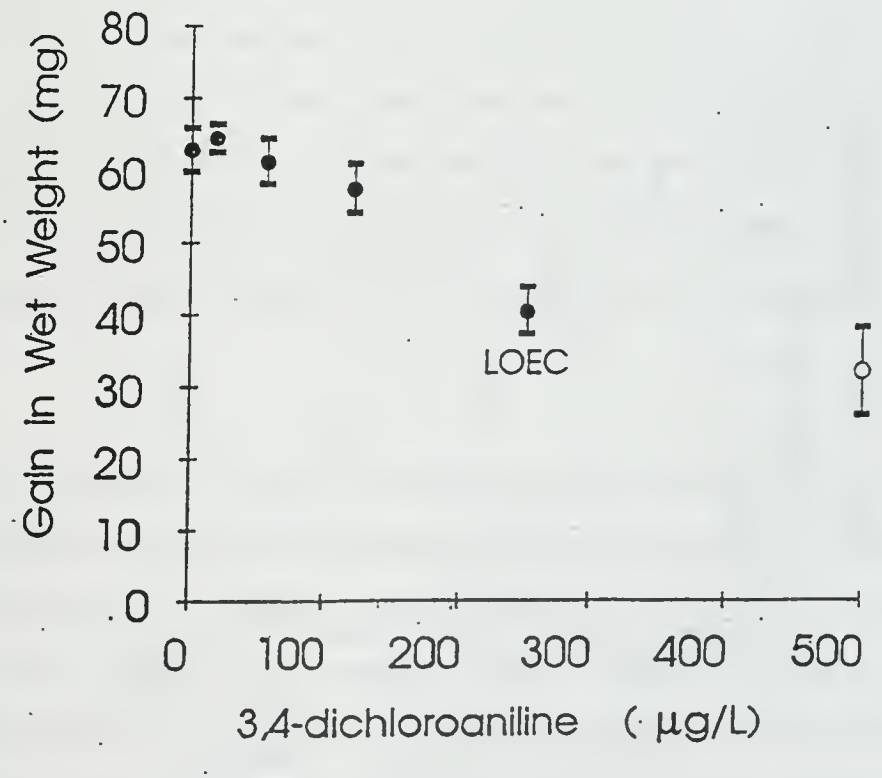

Fig. 6. Dose/response curve (mean ± s.e.) showing wet weight gain of rainbow trout late sacfry to fed swim-up fry exposed to nominal concentration 3,4-DCA in the preliminary test for 12 days. ⊼ indicates invalid group mean wet weight gain due to edema.

None of the fish in the lower concentration groups were exophthalmic or visibly swollen. Data from these groups and the adjusted group 5 data were considered valid. The LOEC and NOEC were 250 µg/L (group 5) and 125 µg/L (nominal concentrations), respectively.

Final 3,4-DCA In this test the nominal concentrations used were in a range increasing by 100 µg/L from 0 - 500 µg/L instead of a series of double dilutions, to provide more information concerning the % body water content in swim-up fry with edema as well as severely delayed yolksac absorption. With this chemical there was no evidence of a non-linear response in concentrations close to the LOEC, (see Table 14 (p.29) and Figure 7), probably due to the relatively large difference, (84 µg/L), between the measured concentrations of the NOEC and LOEC (groups 2 and 3). However, Figure 7 clearly shows a different kind of non-linear response (ie. the flattened dose/response curve) in the two highest concentration groups, indicating a change in toxic mechanism in groups 5 and 6 compared to the lower concentration groups.

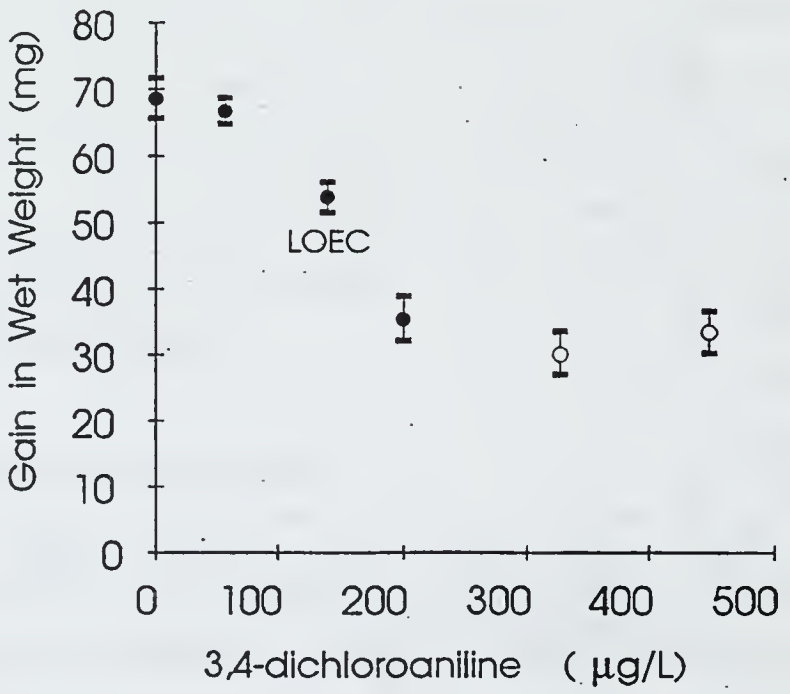

Fig. 7. Dose/response curve (mean ± s.e.) showing wet weight gain of rainbow trout late sacfry to fed swim-up fry exposed to nominal concentration 3,4-DCA in the final test for 12 days. ⊖ indicates invalid group mean wet weight gain due to edema.

The severity of the narcotic effect of DCA on feeding appears to have reached an asymptote in groups 4, 5 and 6 as the wet weight gains were similar in all three groups (see Table 14 (p.29) and Figure 7), but observations on feeding onset and quantity, and the appearance of the fish do not support this conclusion. Feeding was noted to be more severely delayed and reduced by exposure to 448 μg/L (group 6) of the DCA test than to 326 μg/L (group 5), with most of the fish apparently not eating, yet the mean wet weight gain in this group was slightly higher than in group 5. These results indicated edema in the group 6 fish. This was confirmed by the swollen appearance of many of the fish noted at the end of the test, and by the significant increase in the group mean % body water content (Table 15 (p.30)). Subsequently, the measured group mean wet weight gain was invalid, and was not included in the growth analysis.

The mean dry weights of groups 5 and 6 (Table 15 (p.30)) also appear to indicate similar growth in the two groups, but again, this was due to invalid growth data. Almost twice the number of fish had a severe decrease in yolksac absorption in group 6 than in group 5 (Table 16 (p.)), and activity in group 6 was severely reduced during the last two days of exposure. Both of these effects would have decreased energy consumption in the group 6 fish, resulting in a conservation of dry weight. Thus, the mean dry weights of groups 5 and 6 are almost identical despite the much greater severity of reduced exogenous feeding observed in the latter.

Some of the toxic effects in group 5 were almost as severe as those in group 6, and much more severe than in group 4. Yolksac absorption was delayed in 50 % of the fish, (Table 16 (p.31)), feeding onset was delayed at least two days, and feeding quantity was greatly reduced throughout the test. However, only two of the fish appeared to be severely edematous, and there was no significant increase in the group mean % body water content. Nevertheless, there was still only a slight decrease in wet weight gain compared to group 4 fish, (see Table 14 (p.29) and Figure 7), indicating edema in group 5 as well as group 6. It is

apparent that moderate edema occurred in many of the group 5 fish, but was not detected by a significant increase in the whole fish % water content due to the equally severe decrease caused by the delayed yolksac absorption. Hence, due to the flattened dose/response curve between groups 4 and 5 despite the greatly reduced feeding in the latter, the group 5 mean wet weight gain was also deemed to be invalid and was excluded from the growth analysis.

In the lower concentration groups, yolksac absorption and feeding were much less severely delayed than in groups 5 and 6, and the whole fish % water content was not significantly affected. The wet weight gains in these groups were therefore valid. However, as in the preliminary DCA test, reduced food consumption was noted in most of the group 4 fish and a few of the group 3 fish by the 4th day of feeding, indicating that extension of the exposure period may have reduced the LOEC.

Using hypothesis tests, the exclusion of the two highest concentration groups did not affect the outcome of this growth test, even though the sensitivity was slightly reduced. The minimum significant difference was reduced from 13.0% (8.9mg) with 6 groups, to 11.3% (7.8 mg) with four groups. In both cases, the LOEC (group 3) and NOEC (group 2) were the same (see Table 14 (p.29)). Their wet weight gains were 21.5% (14.8mg) and 2.8% (1.9mg) below the control, respectively.

The severely reduced feeding and activity, the swollen appearance, and the reduction in oxygen consumption during the last two days of exposure of the group 6 fish, show that mortality was imminent in the majority of fish in this group, and would undoubtedly have occurred if exposure to this chemical had been continued for a few days. Similarly, the severity of the effects in some of the group 5 fish indicate that they would also have died if the exposure period had been slightly extended. In these circumstances, the test should be repeated using a range of dilutions up to the highest concentration yielding all valid growth

data, and extending the exposure period by approximately one to two weeks[7] before attempting to estimate concentrations likely to cause toxic effects in chronic exposures. In those tests where reduced feeding by the end of the test indicates that the test should be prolonged, the extended period of exogenous feeding will allow the dry weights to be increased sufficiently to overwhelm any effects of delayed yolksac absorption. Dry weights can then be used as the growth parameter so that any edema will no longer confuse interpretation of the growth data. However the fish must still be wet weighed before the test initiation.

The reproducability of this test is shown by the results of this DCA test and those of a later DCA test[4] in which smaller fish had to be used (mean initial weight = 64.2 ± 2.8 mg). In both cases the nominal concentration of the LOEC was 200 μg/L. However, comparison of the appearance of the test fish with that of the controls at the end of the test was sometimes more difficult with the smaller fish.

[7]Adema and Vink (1981) have shown that extension of fish early life stage tests from one to three months using 3,4-DCA makes little difference to the test sensitivity.

Statistical Analysis

The non-linearity in the growth results from the sublethal tests reported here show some of the difficulties that could arise if analysing data from rainbow trout sacfry to swim-up fry ELS growth tests using linear regression or linear interpolation methods of analysis. The data should not be transformed when the non-linearity of the responses is due to a physiological or behavioural response to the toxicant. Hence, the advantage of being able to calculate confidence intervals when using these methods is lost as there is little confidence in them when part of the data is non-linear. Furthermore, data transformation makes the data non-parametric, thereby reducing the sensitivity of statistical analyses and the use of the test in estimating concentrations likely to cause chronic effects. Linear regression analysis is the obvious choice when using a large concentration range, as with most acute tests. However, establishing a single endpoint such as IC_{25} is not necessarily suitable for all endpoints, nor for all life stages using the same endpoint, nor for all species. From the results shown here, IC_{20} would be more suitable for growth in the rainbow trout late sacfry and swim-up fry life stages whereas IC_{25} is probably preferable for juveniles and older lifestages. In this sublethal growth test, where the test concentrations should be in a range not greater than the incipient 12 day mortality level and the data are rarely monotonic, hypothesis testing is the suitable approach for analysis of short-term, subchronic growth data using a sensitive endpoint. The minimum significant weight difference from the controls, calculated in the Dunnett and Bonferroni tests, signifies the precision of the test. One of these tests should always be used in conjunction with other hypothesis tests which may be required, eg. Williams' test for some effluents or Tukey's test for site specific receiving water studies. The % differences of the LOEC and NOEC from the control can be used to calculate ICx values for growth in guideline development.

TEST SENSITIVITY COMPARED TO OTHER TESTS

Copper

The LOECs of the short term ELS tests for copper in moderately hard water and very soft water (<12 μg/L at hardness = 135 mg/L and <10 μg/L at hardness = 10 mg/l), are less than those obtained in this laboratory for fathead minnows using the 7 day larval growth test adapted for use in Ontario, (Neville, 1989), (LOEC = 30μg/L, NOEC = 10μg/L at hardness = 135mg/L; LOEC = 10μg/L, NOEC = 3μg/L at hardness = 50 μg/L, unpublished data). Furthermore, they are lower than the LOEC for copper reported by McKim et al.(1978), who exposed the eyed embryo to early juvenile stage for 47 days in moderately soft water (hardness = 48mg/L). The LOEC was 31.7 μg/L, which caused a growth reduction of ≈ 75%. There was a growth reduction of ≈ 30% at 12 μg/L but under the experimental conditions at that time this was not found to be significant.

The similarity of the 47 day ELS test results and the results of this short-term ELS test is not surprising. The initial toxic action of copper in fish is the reduction in oxygen uptake at the respiratory surface as the respiratory epithelium becomes damaged. Subsequently, energy reserves and liver and kidney tissues are impaired. During embryo-larval development the primary respiratory site changes from the fine cutaneous epithelium covering underlying blood vessels in the embryo and yolksac, with a gradual transition to the gills in sacfry and swim-up larvae. The secondary lamellae in the gills of rainbow trout are already developed at hatch (Morgan, 1974) and gradually increase in oxygen uptake capacity as the yolksac is absorbed, the thickness of the cutaneous epithelium increases, and larval activity increases. Hence, copper can be absorbed at all stages of larval development but the rate increases in conjunction with increased activity and oxygen requirements. At the same time, with increased activity the effects of copper toxicity on growth become more apparent. Therefore, the late sacfry to early swim-up stages are the most effective for use in a short-term salmonid ELS growth test with copper, and other toxicants with similar mechanisms of uptake.

SDS

The short-term growth test with sodium dodecyl sulphate (SDS) was carried out during the preliminary stages of test development before techniques to increase the sensitivity had been perfected. Also, a wide range of concentrations was used with only 3 fish per concentration. Nevertheless, the lowest nominal concentration causing reduced growth (0.3 mg/L) was approximately 1/20 of the SDS 96h LC50 for rainbow trout (4.3 - 8.5 mg/L, Doe and Wells, 1978). Compared to other short-term "chronic" toxicity tests, the sensitivity was approximately 20 times that of the 7 day fathead minnow larval growth and survival test (LOEC = 9.2mg/L, NOEC = 4.6mg/L, Pickering, Q.H., 1988) and 100 times that of the EC50 for reproductive success in the *Ceriodaphnia dubia* three brood test (EC50 = 36mg/L, Cowgill and Milazzo, 1991). The sensitivity of the sacfry and swim-up stages of rainbow trout to sodium dodecyl sulphate is readily apparent.

Final 2,4,5-TCP

There are few literature reports on the subchronic or chronic toxicity of 2,4,5-TCP to aquatic biota, and none were found using salmonids. However, Hodson et al (1991) reported regression estimates of chronic toxicity of 0.32 μM (52 μg/L) for 2,4-dichlorophenol and 0.09 μM (24 μg/L) for pentachlorophenol in flowthrough rainbow trout growth tests using ELS from fertilisation to 28 days post swim-up at pH 7.9-8.1. Since chlorophenol toxicity increases with chlorination (Kovacs et al, 1993) as well as with acidity, an LOEC of <34 μg/L (0.17 μM) for 2,4,5-trichlorophenol at pH 7.4-7.8 indicates a similar test sensitivity between this short-term ELS growth test and the traditional 90 day ELS test. In comparison with other species, Norberg-King (1989) used TCP (reported as 2,4,5-TCP in Aquire) among other chemicals to evaluate the fathead minnow 7-day growth and survival test, (Norberg and Mount, 1985). The LOEC and NOEC were 684 μg/L and 361 μg/L respectively; the pH was not given. In a study on the bioaccumulation of phenols, Call et al. (1980) exposed juvenile fathead minnows to 50 μg/L 2,4,5-TCP for 28 days. Fish survival and growth were not affected at this concentration. The dilution water pH was 7.36 - 7.62 but the pH during the test was not reported. Also

using warmwater fish, Nielson, (1990), reported an LOAEL[8] of 150 μg/L in a 14 day exposure of embryo/larval zebrafish to 2,4,5-TCP at pH 7.2. The sensitivity of the rainbow trout test at pH 7.4-7.7 reported here, where the lowest concentration used (34 μg/L) was still toxic, was at least twice, and possibly greater than 20 times that of fathead minnows, and at least 5 times greater than zebrafish.

Final 3,4-DCA

Juvenile rainbow trout (3-5g) have been used in 14 and 28 day tests with 3,4-DCA, (Crossland, 1990). The fish were in the exponential growth phase, therefore specific growth rates were used as the endpoint. After 14 days exposure the LOEC and NOEC were 210 and 120 μg/L, nearly twice that for the sacfry to swim-up fry test reported here, (139 and 55μg/L), but after 28 days they were 3 to 4 times lower, (39 and 19 μg/L, respectively). At the sacfry and swim-up fry stage, the reduction in feeding evident by the fourth day in the groups lower than the LOEC suggests that the LOEC and NOEC would also be reduced after extending the exposure period by 1 - 2 weeks and using a concentration range further below the incipient acute mortality level. A round-robin test of this procedure would be useful in comparing the sensitivity of these two tests. Using Crossland's procedure in a round-robin 28 day test with eleven participants, the mean and range of LOECs and NOECs were 325 (100-600) μg/L and 95 (30-200) μg/L, respectively, (Ashley et al., 1990). However, the sensitivities of the early juvenile test and the sacfry to swim-up fry test appear to be fairly similar using DCA. With this chemical, both of these short-term coldwater fish tests appear to be less sensitive than a 28 day, complete early life stage, warmwater fish test; Call et al. (1987) reported an LOEC of 7.1 μg/L using a 28 day fathead minnow fertilisation to early juvenile test.

[8]Lowest observed adverse effect level, a more precise term for the LOEC.

CONCLUSIONS

The test concentrations used in this short-term, sublethal, early life stage growth test should be below 12 day incipient mortality levels for rainbow trout fry and sacfry, to avoid invalidating growth data in the higher concentration groups due to edema. However, if edema should occur, it can be readily identified by the shape of the dose response curve which will appear to be reaching an asymptote after showing decreasing wet weight gains with increasing toxicant concentration in the middle concentration range. As well, exogenous feeding will be absent or severely reduced in the edematous group(s) compared to lower concentrations and the controls. Identifying edema by measuring dry weights as well as the wet weights to calculate the whole fish % water content not only increases the cost of the test, but also will not identify edema in swim-up fry if yolksac absorption has been severely delayed and the edema is only moderately severe. Dry weight data should not be used for growth analysis in this test since it decreases during the sacfry stage, and, if feeding has been reduced by toxicant exposure, is often less sensitive than wet weight gain during the first few days of exogenous feeding.

The non-linear, significantly reduced, growth responses often seen in lower concentration groups close to the LOEC, and occasionally seen in the higher concentration groups if edema has occurred, are valid biological responses, therefore the data should not be transformed to make it suitable for analysis by linear regression. In this test protocol, avoiding large concentration ranges and analysing the untransformed data with appropriate hypothesis tests will provide a more reliable estimate of growth than linear regression. The typical group responses described above illustrate the precision and sensitivity of LOEC and NOEC data when using a sub-incipient mortality range of test concentrations with wet weight gain as the growth end-point. The minimum significant (growth) difference, (MSD), and the % reductions caused by the LOEC and NOEC, will show the test sensitivity. In the experiments reported here the mean MSD for growth was 14.4% $\pm$ 3.6 ($n = 5$), and the growth reduction caused

by the LOEC and NOEC groups was approximately 20% and 5% respectively, using a series of double dilutions and a 5% level of significance, therefore with this test protocol the LOEC and NOEC could be used to calculate an EC20 and EC5 for estimation of chronic effects in the field. An EC50 for growth in short-term tests is too close to rainbow trout early life stage mortality to be a useful chronic value.

The results from these individual chemical tests show that use of the late sacfry to early fry lifestage in a sensitive, cost effective, sublethal, short-term ELS rainbow trout growth test allows maximum toxicant uptake as well as maximum expression of toxicant effects for estimating chronic ELS exposure effects of metals, surfactants, and organic chemicals in coldwater fish. Limiting the test to 12 days, with 12 individually exposed fish per concentration, reduces costs to the minimum with little loss of sensitivity compared to traditional early life stage tests. For a few classes of chemicals with lower absorption rates, including non-polarised forms of organic chemicals with narcotic properties such as 3,4-DCA, the exposure period may need to be extended to approximately 21 days.

ADVANTAGES of using the late sacfry to early exogenously feeding swim-up fry stage of rainbow trout in a short-term early life stage growth test for coldwater fish.

- use of optimal early life stages for toxicant absorption (sacfry) and expression of effects (swim-up fry)
- sub-incipient mortality toxicant concentrations used - no carrier solution required
- 12 day exposure period for most chemicals - (organic chemicals with low absorption rates, including non-polarised chemicals with narcotic properties, may require up to 21 days exposure.)
- 12 individually weighed and exposed fish per concentration, therefore 12 true replicates per concentration.
- simple test maintenance during the 6-7 days of sacfry exposure due to low rates of oxygen consumption and no feeding required.
- individual exposure chambers, and use of live food after swim-up, avoids problems with initiation of feeding in the swim-up fry.
- individual growth not affected by competition for food.
- use of a static renewal exposure system and individual sections in each exposure tank allows easy observation of any gross abnormality in swimming behaviour, relative rate of yolksac absorption, and relative amount and age at onset of food consumption and faecal production, compared to the controls.
- the simple renewal system allows quick and easy transference of fish to clean exposure tanks, avoiding problems with buildup of faecal waste and uneaten food during the swim-up fry stage.
- small volume of dilution water and low concentration stock solutions required, therefore low volume of toxic waste produced.
- economical in terms of time, space, and number and cost of fish used per test, and in the number of effluent or receiving water samples required when the test is used for the detection of chronic levels of toxicity in the field.

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APPENDIX

Table A1 Wet weight, dry weight and % water content of rainbow trout sacfry from hatch to 4d post swim-up, without feeding.

Age (days)	Wet weight (mg) x ± s.d.(n)	Dry weight (mg) x ± s.d.(n)	Water content (%) x ± s.d.(n)
1	95.5 ± 7.9(4)	34.7 ± 2.4(4)	63.7 ± 0.9(4)
2	93.8 ± 9.1(5)	34.1 ± 3.6(5)	63.7 ± 0.6(5)
3	96.2 ± 9.4(5)	34.5 ± 3.8(5)	64.2 ± 0.9(5)
6	103.5 ± 4.6(5)	34.9 ± 1.6(5)	66.3 ± 0.6(5)
8	110.0 ± 5.5(4)	35.9 ± 1.7(4)	67.3 ± 0.7(4)
9	113.4 ± 3.3(5)	36.2 ± 4.0(5)	68.1 ± 0.4(5)
10	117.3 ± 6.1(5)	36.0 ± 2.5(5)	69.3 ± 1.3(5)
13	128.7 ± 7.3(5)	37.0 ± 2.6(5)	71.3 ± 0.8(5)
14	127.0 ± 8.8(5)	35.3 ± 2.9(5)	72.2 ± 0.8(5)
15	129.3 ± 8.8(5)	34.6 ± 2.8(5)	73.3 ± 0.6(5)
16	133.4 ± 5.9(5)	34.9 ± 2.2(5)	73.9 ± 0.7(5)
17	137.0 ± 7.6(5)	34.2 ± 1.8(5)	75.0 ± 0.5(5)
20	136.3 ± 11.5(5)	31.5 ± 3.1(5)	76.8 ± 1.8(5)
21	142.5 ± 5.1(5)	32.3 ± 1.8(5)	77.3 ± 0.7(5)
22	149.9 ± 3.9(5)	32.4 ± 1.6(5)	78.4 ± 0.6(5)
23	148.8 ± 5.6(5)	31.0 ± 1.9(5)	79.1 ± 0.7(5)

Table A2. Dilution Water Quality at 25°C.[1]

Conductivity	321 μMho/M^3 @ 25°C
Hardness	131.0 mg/L as $CaCO_3$
Calcium	38.5 mg/L
Magnesium	8.5 mg/L
Sodium	12.3 mg/L
Potassium	1.6 mg/L
Alkalinity	89.7 as $CaCO_3$
pH	7.9
Fluoride	1.1 mg/L
Chloride	24.7 mg/L
Sulphate	29.0 mg/L
Total nitrogen	0.160 mg/L
Total ammonium	0.004 mg/L[2]
Total nitrate	0.380 mg/L
Nitrite	0.0010 mg/L[2]
Dissolved organic carbon	1.5 mg/L
Dissolved inorganic carbon	20.6 mg/L
Total residual chlorine	<30 μg/L
Copper	0.0012 mg/L[2]
Nickel	<0.002 mg/L[3]
Lead	<0.005 mg/L[3]
Zinc	0.0026 mg/L
Iron	0.022 mg/L[2]
Cadmium	<0.0002 mg/L[3]
Chromium	<0.0005 mg/L[3]

[1] MOEE, Laboratory Services Branch [2] Trace [3] None detected